

MICROSCOPIC OBSERVATION OF A ROOT-ADHERING FACTOR *Avin_16040* IN *Azotobacter vinelandii* AND *Escherichia coli* DURING ASSOCIATION WITH RICE ROOT

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ABSTRACT

Microbial adherence to plant root is the initial step in a beneficial plant-microbe interaction. Quantitative RT-PCR analysis deduced the *Avin_16040* gene showed upregulated expression when *Azotobacter vinelandii* was adhered to the rice root. By transforming the full-length *Avin_16040* gene into a heterologous host *Escherichia coli*, the recombinant clones displayed filamentous cell shapes in contrast to the rod-shape of wild type cells. Besides full-length gene insert, some *E. coli* clones were detected to contain truncated *Avin_16040* gene inserts but still shape-shifted to filamentous cells. Further analysis by DNA sequencing revealed the shape-shifting *E. coli* clones contained 3'-end truncated *Avin_16040* gene, while *E. coli* clones containing the 5'-end truncated *Avin_16040* gene remained rod-shaped. The cell surface topographies of *A. vinelandii* and *E. coli* cells in the presence and absence of *Avin_16040* gene and in association with rice root adherence were analysed using atomic force microscopy.

Keywords: *Avin_16040*, *Azotobacter vinelandii*, *Escherichia coli*, AFM

INTRODUCTION

Azotobacter vinelandii is a plant-growth-promoting bacterium commonly found in soil. It is well known as a dinitrogen fixer, as well as a plant growth hormone producer. Besides, *A. vinelandii* has a long research history of biosynthesizing the extracellular polysaccharide alginate, the intracellular polyester poly- β -hydroxybutyrate (PHB) and siderophores which are compounds that were reported to have multiple biotechnology and biomedical applications. These applications include alginate for control release of medical drugs (Yao *et al.*, 2009) and as food additives (thickener, stabilizer, gelling agent and emulsifier), polyhydroxybutyrate (PHB) for development of biodegradable and biocompatible thermoplastics (Diaz-Barrera & Soto, 2010), and siderophores as drug delivery (Möllmann *et al.*, 2009), antimicrobial (Upadhyay & Srivastava, 2008) and soil bioremediation agents (Braud *et al.*, 2009).

Plant-growth-promoting rhizobacteria (PGPR) can improve the overall development of plants by increasing its root and shoot yields (Zakry *et al.*, 2010). As a PGPR, *A. vinelandii*'s N₂ fixation has been extensively studied since 1960s. Other PGPR characteristics most commonly researched into are its ability to produce plant growth hormone and amino acids. Torres-Rubio *et al.* (2000) reported that *A. vinelandii* isolated from a rice rhizosphere produced the highest concentration of indole-3-acetic acid (IAA) in comparison to the other isolates residing at the same rhizosphere, namely *A. chroococcum*, *Pseudomonas aeruginosa*, *P. putida* and *Serratia* sp. Rodelas *et al.* (1999) and Pozo *et al.* (2000) discussed the production of plant-growth-promoting amino acids by *A. vinelandii* and *A. chroococcum*. *A. vinelandii* ATCC 12837 was also reported to colonize the rhizospheres of cereal (Shimshick & Hebert, 1979), rice (Maudinas *et al.*, 1981; Torres-Rubio *et al.*, 2000), wheat (Naz *et al.*, 2012) and hot pepper (Husen, 2005). This non-endosymbiont root association has brought about supply of biologically fixed N₂ to the plant,

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enhanced soluble P availability, antibiosis against pathogenic microorganisms, as well as supplies of plant-growth-promoting hormones, vitamins and amino acids to the plant (Okon & Itzigsohn, 1995; González López *et al.*, 1999; Revillas *et al.*, 2005). Concomitantly, root-colonizing *A. vinelandii* can incorporate readily the plant root exudates which may contain sugars and organic acids.

Recently, we reported that a surface layer protein *Avin_16040* was involved in the adherence of *A. vinelandii* ATCC 12837 cells to rice root (Liew *et al.*, 2015). In the same study, we observed that the heterologous host *E. coli* showed increased adherence to rice root after it was transformed with *Avin_16040* gene. This paper aims to impart differing cell surface topographies of *A. vinelandii* and *E. coli* cells in the presence and absence of *Avin_16040* gene in relation to root adherence. For this purpose, atomic force microscopy (AFM) was used. Besides, DNA cloning of *Avin_16040* gene in *E. coli* will be discussed by emphasizing that the *E. coli* clones which contain 3'-end truncated *Avin_16040* gene also displayed "filamentous cell" effect as the *E. coli* clones which contained full-length *Avin_16040* gene.

MATERIALS AND METHODS

Bacterial strains, plasmid and culture conditions

Azotobacter vinelandii Lipman ATCC 12837 was obtained from the American Type Culture Collection (ATCC). Deletion mutant *A. vinelandii* Δ *Avin_16040* was described previously by Liew *et al.* (2015). Both bacterial strains were maintained and grown in Burk's medium containing 2% (w/v) sucrose. Liquid cultures were agitated continuously at 200 rpm, $25 \pm 2^\circ\text{C}$, for up to 5 days. Agar medium was solidified with 2% Agar Bacteriological No.1 (Oxoid, UK). *A. vinelandii*-root interaction was performed in altered Murashige and Skoog (1967) medium as described before (Liew *et al.*, 2015). *E. coli* DH5 α was cultivated in Nutrient Broth (NB) (Merck, USA) for overnight at $37 \pm 2^\circ\text{C}$.

Sample preparation and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

O. sativa MR 219 seeds were surface sterilized and germinated as described by Liew *et al.* (2015). Axenic roots of *O. sativa* MR 219 were interacted with *A. vinelandii* ATCC 12837 wild type in three systems; (1) adventitious roots were aseptically excised from the rice seedlings with scalpel blade and cut into short fragments of approximately 1 cm before they were mixed separately with ATCC 12837 cells at 10^7 CFU/mL concentrations in the altered-MS medium, (2) adventitious roots were

shredded and vortexed vigorously in the altered-MS medium, after which shredded roots were harvested and re-suspended in fresh medium containing ATCC 12837 cells as described for 1 cm root fragments, (3) root extract (supernatant) was interacted separately with ATCC 12837 cells. Root-microbe interaction was performed for 2 hrs at static condition. The positive control was constructed by mixing the ATCC 12837 cells with the roots of the rice seedlings. Root-adhered ATCC 12837 cells were lysed immediately in cell lysis solution and processed for RNA extraction. RNAs were also prepared from the free-floating cells (not adhered to roots) in the root-microbe interaction systems as well as non-root-interacted bacterial culture (negative control). To verify the expression of *Avin_16040*, root-adhered ATCC 12837 cells were analysed after 0 hr, 10 min, 20 min, 30 min, 1 hr, 2 hrs, 4 hrs, 8 hrs and 24 hrs of root-microbe interaction. RNA extraction, reverse transcription and qRT-PCR were performed according to Liew *et al.* (2015).

DNA manipulations and analyses

The DNA fragment encoding the *Avin_16040* gene was ligated to the plasmid vector pJET1.2/blunt (Fermentas, Lithuania) before the mixture was transformed into *E. coli* DH5 α . Bacterial cells were smeared on glass slides and observed under Primo Star upright microscope (Zeiss, USA). DNA inserts were amplified by PCR. *E. coli* clones carrying different DNA insert sizes were selected for further analysis. DNA sequencing was performed commercially.

AFM

Atomic force microscopy (AFM) was conducted using JPKNanoWizard II system (JPK Instruments AG, Germany) to display bacterial cell structure and cell surface topography. Bacterial smears were used for the purpose. The bacterial smears were prepared according to Liew *et al.* (2015).

RESULTS AND DISCUSSION

The bacterial strain *A. vinelandii* ATCC 12837 was studied during its interaction with *O. sativa* MR 219 root and a hypothetical protein *Avin_16040* was identified via a 2-dimensional gel electrophoresis – tandem mass spectrometry (2DE-MS/MS) method (Liew *et al.*, 2015). The protein was found to show an elevated expression level during close contact with *O. sativa* MR 219 roots. An *Avin_16040* deletion mutant was then generated, which revealed some interesting aspects on the functions encoded by the protein coding gene.

In this paper, we present additional data on the hypothetical gene coding for *Avin_16040* as well as on the characteristics of its deletion mutant *A. vinelandii* Δ *Avin_16040*, especially in relation to the root adherence function. Preliminarily, the *in vitro* interaction between *A. vinelandii* and *O. sativa* was conducted by subjecting the bacterial cells to roots of the *O. sativa* seedlings. To explore whether the root (physical effect) or root exudates (chemical effect) caused the elevated expression of *Avin_16040*, *A. vinelandii* was interacted with roots that were cut into short fragments of 1 cm in length, shredded roots, and root extracts. Root extract was obtained by separating the medium supernatant from the shredded root debris after vigorous vortexing. Relative to the cells at 0 hour, *Avin_16040* gene showed an upregulated expression in the bacterial cells which resided on *O. sativa* roots of the seedlings, as well as on the root fragments of 1 cm in length (Figure 1). No significant change in expression levels was detected in the interactions with root extract and shredded roots. The results indicated that the elevated expression of *Avin_16040* gene was caused by *A. vinelandii*'s adherence to root (physical effect). Further analysis at selected time points revealed the *Avin_16040* gene responded almost immediately to the root (fragment)-microbe interaction. Figure 2 shows that its expression started to escalate after only 10 min interaction with roots. By supplementing nitrogen (1.65 g L⁻¹ ammonium nitrate) to the interaction medium, *Avin_16040* gene showed lower expression level in general. The ability to colonize plant root is an important characteristic of plant-beneficial

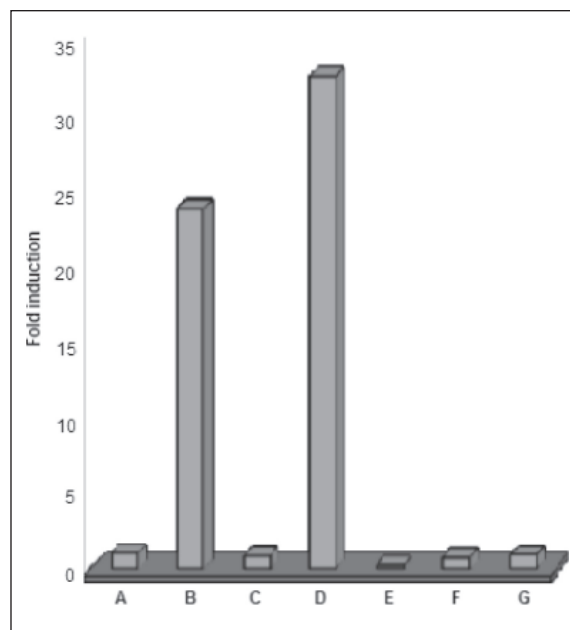


Fig. 1. Comparison of *Avin_16040* gene expression extracted from different *O. sativa* roots – *A. vinelandii* interaction conditions. Bacterial cells were interacted with roots for 2 hrs at static condition. The results showed *Avin_16040* gene was upregulated in *A. vinelandii* attached to *O. sativa* roots. A, *A. vinelandii* cells at 0 hr, B, root-attached bacterial cells after 2-hr interaction with *O. sativa* root attached to seedlings, C, free-floating bacterial cells after 2-hr interaction with *O. sativa* root attached to seedlings, D, root-attached bacterial cells after 2-hr interaction with cut *O. sativa* root fragment, E, free-floating bacterial cells after 2-hr interaction with cut *O. sativa* root strands, F, bacterial cells after 2-hr interaction with *O. sativa* root extract, G, bacterial cells after 2-hr interaction with *O. sativa* roots shredded with scalpel blade.

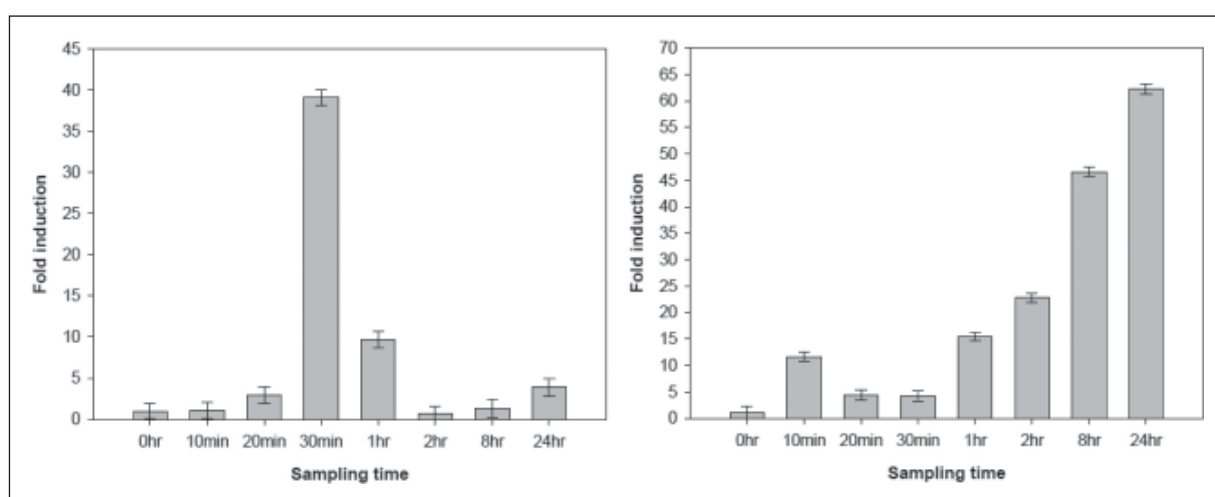


Fig. 2. Quantitative RT-PCR analysis of *A. vinelandii* ATCC 12837 during root adherence in altered Murashige and Skoog (1967) medium containing nitrogen (left) and devoid of nitrogen (right). The expression level of *Avin_16040* gene was normalized against the 16S rRNA gene (Liew *et al.*, 2015). In the medium containing nitrogen, *Avin_16040*'s expression fluctuated where it spiked upon 30 min of *A. vinelandii* – *O. sativa* root interaction before decreased, but detected slight increase after 24 hrs of interaction. In contrast, the expression of *Avin_16040* gene increased steadily after 30 min of interaction until the experiment was stopped at 24-hr.

bacteria. This close proximity between microbe and plant root implied that the microbe could directly affect the performance of plant via mutual exchange of signal molecules such as phytohormones and nutrients, as well as sugars and amino acids (Brechenmacher *et al.*, 2010). This association resembles a symbiotic relationship.

Using AFM, the cell shapes and structures of *A. vinelandii* during interaction with *O. sativa* roots were observed (Figure 3). In general, both Δ *Avin_16040* deletion mutant and ATCC 12837 strains showed the formation of “thread-like” networks during root adherence. However, the deletion mutant displayed “grainy” motif which is narrower in width than the “filamentous” motif of the ATCC 12837 wild type. In addition to the “grainy” motif, the root-adhered deletion mutant also displayed “patches” motif which were most possibly amassed from groups of cells as viewed under the compound microscope (Fig. 3E). The different motif formation directly reflected the decreased root adherence capacity of Δ *Avin_16040* which was reported previously by Liew *et al.* (2015). AFM analysis of the non-root-interacted *A. vinelandii* cells revealed Δ *Avin_16040* cells were generally bigger than the ATCC 12837 cells. By performing electron microscopy analysis, Shimshick and Hebert (1979) reported that *A. vinelandii* ATCC 12837 colonizes plant root in both monolayer and multilayer formats until 10^9 cells per g root. Even though the majority of ATCC 12837 cells adhered to root individually, some adhered in patches of several hundred cells with considerable overlaps (Shimshick & Hebert, 1979).

The DNA fragment of *Avin_16040* gene was cloned in pJET1.2/blunt plasmid vector and transformed into *E. coli* DH5 α . By performing PCR analyses, recombinant *E. coli* clones were found to contain several DNA insert sizes and were further analysed. DNA sequencing analysis showed that partial *Avin_16040* genes with 3'-end truncation and 5'-end truncation were cloned (Figure 4). The cause and mechanism that brought about the insert size variation was unknown. However, it provided additional information which was useful in the genetic mapping of S-layer proteins. Translation of a recombinant protein especially by a high copy number plasmid might impose a metabolic burden that decreases the growth rate of bacterial host and affects plasmid instability (Bentley *et al.*, 1990; Birnbaum & Bailey, 1991). In such an occurrence, truncation of the recombinant gene might be a successive effect. The recombinant clones carrying different *Avin_16040* gene fragments were selected for cell morphology analysis using a compound microscope. The *E. coli* clones (represented by clone 58) which were transformed with the full-length *Avin_16040* gene (1,368 bp) had cell shapes which

were filamentous (Figure 5). Interestingly, the *E. coli* clones containing 3'-end truncated *Avin_16040* genes of 1,256 bp (clone 84) and 346 bp (clone 27) in length were also displaying filamentous cell morphology. In contrast, an *E. coli* clone (clone 10) which contained 5'-end truncated *Avin_16040* genes showed no change in its cell morphology. Cloning of 5'- and 3'-end truncated surface layer gene fragments and the expression of N-terminal protein segment (C-terminal or 3'-end gene truncation) are crucial for cell surface display of functional polypeptides (Knobloch *et al.*, 2012). The same study also showed that insertion of functional peptide sequences or single amino acids had no or only slight effect on the formation of S-layer protein sheets. In contrast, the C-terminally truncated S-layer protein has lost its cell surface display function. Typically, the S-layer structural protein consisted of two functional regions. The N-terminal region was involved in the attachment of S-layer subunit to the underlying cell wall (transmembrane) while the middle and C-terminal region was involved in S-layer assembly (Åvall-Jääskeläinen *et al.*, 2008). Our observation matches that of Knobloch *et al.* (2012) showing that the 5' gene segment of *Avin_16040* was crucial for its expression as implicated by the formation of filamentous cells. It is also worth mentioning that a 5' gene fragment of as small as 346 bp was able to impose *E. coli* with the shape-shifting effect. Finally, we used AFM to display the cell surface topography of *E. coli* cells which acquired the complete *Avin_16040* gene of 1,368 bp. The images in Figure 6 are in agreement with previous report (Liew *et al.*, 2015) that *Avin_16040* over-expresses during its adherence to the rice root. Both Figure 6C and 6D demonstrated elongated *E. coli* cells expressing the *Avin_16040* gene relative to the untransformed cells. However, the over-expression of *Avin_16040* were particularly demonstrated by the thickened cell as displayed by the recombinant *E. coli* cells adhering to rice root (Figure 6D). Besides, the root-adhered recombinant cells were enlarged due to its association with rice root. It is interesting to observe that the non-transformed *E. coli* DH5 α cells were enlarged and thickened during association with rice root (Figure 6B). Wheeler *et al.* (2015) has provided evidence that peptidoglycan hydrolases play important role in bacterial cell enlargement through hydrolysis and expansion of the peptidoglycan in the bacterial cell wall, exemplified by *Staphylococcus aureus*. Overall, our study provides additional information for use in the future research works involving biosynthesis, assembly and genetic inferences of the S-layer proteins. Besides, our findings may contribute to its design and applications as cell surface display for the various S-layer fusion proteins.

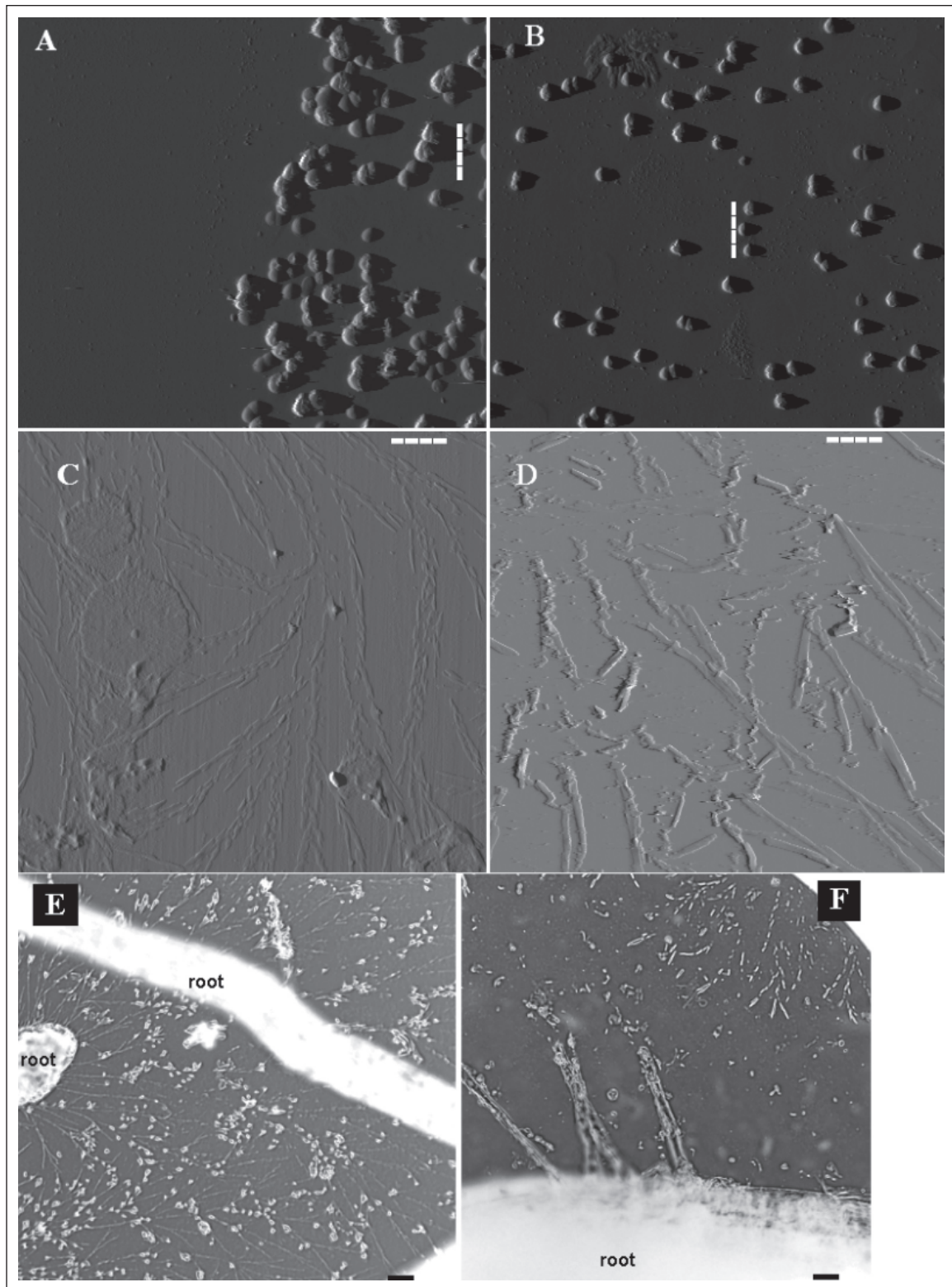


Fig. 3. Observation of *A. vinelandii* ΔA_{vin_16040} and ATCC 12837 cells adhered to rice roots. A-D showed the cell surface topographies of ΔA_{vin_16040} and ATCC 12837 as displayed by AFM, while E-F showed the compound microscopic observation of root-adhered ΔA_{vin_16040} and ATCC 12837 cells observed at 400x magnification. In general, the “non-root-interaction” deletion mutant ΔA_{vin_16040} cells (A) displayed bigger cells than the wild type ATCC 12837 cells (B). During root adherence, the mutant cells showed grain-like network and patches (C, E), different from the filament structures of the wild type cells (D, F). The different root-adherence ability of ΔA_{vin_16040} and ATCC 12837 cells that was previously reported by Liew *et al* (2015) could be coincided with the observation. A, ΔA_{vin_16040} cells (non-root-interaction), B, ATCC 12837 cells (non-root-interaction), C, ΔA_{vin_16040} cells adhered to rice root surface, D, ATCC 12837 cells adhered to rice root surface. Scale bar = 10 μ m.

C1	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
C2	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300
C3	310	320	330	340	350	360	370	380	390	400	410	420	430	440	450
C4	470	480	490	500	510	520	530	540	550	560	570	580	590	600	610
C5	630	640	650	660	670	680	690	700	710	720	730	740	750	760	770
C6	790	800	810	820	830	840	850	860	870	880	890	900	910	920	930
C7	950	960	970	980	990	1000	1010	1020	1030	1040	1050	1060	1070	1080	1090
C8	1120	1130	1140	1150	1160	1170	1180	1190	1200	1210	1220	1230	1240	1250	1260
C9	1290	1300	1310	1320	1330	1340	1350	1360	1370	1380	1390	1400	1410	1420	1430
C10	1460	1470	1480	1490	1500	1510	1520	1530	1540	1550	1560	1570	1580	1590	1600
C11	1630	1640	1650	1660	1670	1680	1690	1700	1710	1720	1730	1740	1750	1760	1770
C12	1790	1800	1810	1820	1830	1840	1850	1860	1870	1880	1890	1900	1910	1920	1930
C13	1950	1960	1970	1980	1990	2000	2010	2020	2030	2040	2050	2060	2070	2080	2090
C14	2120	2130	2140	2150	2160	2170	2180	2190	2200	2210	2220	2230	2240	2250	2260
C15	2290	2300	2310	2320	2330	2340	2350	2360	2370	2380	2390	2400	2410	2420	2430
C16	2460	2470	2480	2490	2500	2510	2520	2530	2540	2550	2560	2570	2580	2590	2600
C17	2630	2640	2650	2660	2670	2680	2690	2700	2710	2720	2730	2740	2750	2760	2770
C18	2790	2800	2810	2820	2830	2840	2850	2860	2870	2880	2890	2900	2910	2920	2930
C19	2950	2960	2970	2980	2990	3000	3010	3020	3030	3040	3050	3060	3070	3080	3090
C20	3120	3130	3140	3150	3160	3170	3180	3190	3200	3210	3220	3230	3240	3250	3260
C21	3290	3300	3310	3320	3330	3340	3350	3360	3370	3380	3390	3400	3410	3420	3430
C22	3460	3470	3480	3490	3500	3510	3520	3530	3540	3550	3560	3570	3580	3590	3600
C23	3630	3640	3650	3660	3670	3680	3690	3700	3710	3720	3730	3740	3750	3760	3770
C24	3790	3800	3810	3820	3830	3840	3850	3860	3870	3880	3890	3900	3910	3920	3930
C25	3950	3960	3970	3980	3990	4000	4010	4020	4030	4040	4050	4060	4070	4080	4090
C26	4120	4130	4140	4150	4160	4170	4180	4190	4200	4210	4220	4230	4240	4250	4260
C27	4290	4300	4310	4320	4330	4340	4350	4360	4370	4380	4390	4400	4410	4420	4430
C28	4460	4470	4480	4490	4500	4510	4520	4530	4540	4550	4560	4570	4580	4590	4600
C29	4630	4640	4650	4660	4670	4680	4690	4700	4710	4720	4730	4740	4750	4760	4770
C30	4790	4800	4810	4820	4830	4840	4850	4860	4870	4880	4890	4900	4910	4920	4930
C31	4950	4960	4970	4980	4990	5000	5010	5020	5030	5040	5050	5060	5070	5080	5090
C32	5120	5130	5140	5150	5160	5170	5180	5190	5200	5210	5220	5230	5240	5250	5260
C33	5290	5300	5310	5320	5330	5340	5350	5360	5370	5380	5390	5400	5410	5420	5430
C34	5460	5470	5480	5490	5500	5510	5520	5530	5540	5550	5560	5570	5580	5590	5600
C35	5630	5640	5650	5660	5670	5680	5690	5700	5710	5720	5730	5740	5750	5760	5770
C36	5790	5800	5810	5820	5830	5840	5850	5860	5870	5880	5890	5900	5910	5920	5930
C37	5950	5960	5970	5980	5990	6000	6010	6020	6030	6040	6050	6060	6070	6080	6090
C38	6120	6130	6140	6150	6160	6170	6180	6190	6200	6210	6220	6230	6240	6250	6260
C39	6290	6300	6310	6320	6330	6340	6350	6360	6370	6380	6390	6400	6410	6420	6430
C40	6460	6470	6480	6490	6500	6510	6520	6530	6540	6550	6560	6570	6580	6590	6600
C41	6630	6640	6650	6660	6670	6680	6690	6700	6710	6720	6730	6740	6750	6760	6770
C42	6790	6800	6810	6820	6830	6840	6850	6860	6870	6880	6890	6900	6910	6920	6930
C43	6950	6960	6970	6980	6990	7000	7010	7020	7030	7040	7050	7060	7070	7080	7090
C44	7120	7130	7140	7150	7160	7170	7180	7190	7200	7210	7220	7230	7240	7250	7260
C45	7290	7300	7310	7320	7330	7340	7350	7360	7370	7380	7390	7400	7410	7420	7430
C46	7460	7470	7480	7490	7500	7510	7520	7530	7540	7550	7560	7570	7580	7590	7600
C47	7630	7640	7650	7660	7670	7680	7690	7700	7710	7720	7730	7740	7750	7760	7770
C48	7790	7800	7810	7820	7830	7840	7850	7860	7870	7880	7890	7900	7910	7920	7930
C49	7950	7960	7970	7980	7990	8000	8010	8020	8030	8040	8050	8060	8070	8080	8090
C50	8120	8130	8140	8150	8160	8170	8180	8190	8200	8210	8220	8230	8240	8250	8260
C51	8290	8300	8310	8320	8330	8340	8350	8360	8370	8380	8390	8400	8410	8420	8430
C52	8460	8470	8480	8490	8500	8510	8520	8530	8540	8550	8560	8570	8580	8590	8600
C53	8630	8640	8650	8660	8670	8680	8690	8700	8710	8720	8730	8740	8750	8760	8770
C54	8790	8800	8810	8820	8830	8840	8850	8860	8870	8880	8890	8900	8910	8920	8930
C55	8950	8960	8970	8980	8990	9000	9010	9020	9030	9040	9050	9060	9070	9080	9090
C56	9120	9130	9140	9150	9160	9170	9180	9190	9200	9210	9220	9230	9240	9250	9260
C57	9290	9300	9310	9320	9330	9340	9350	9360	9370	9380	9390	9400	9410	9420	9430
C58	9460	9470	9480	9490	9500	9510	9520	9530	9540	9550	9560	9570	9580	9590	9600
C59	9630	9640	9650	9660	9670	9680	9690	9700	9710	9720	9730	9740	9750	9760	9770
C60	9790	9800	9810	9820	9830	9840	9850	9860	9870	9880	9890	9900	9910	9920	9930
C61	9950	9960	9970	9980	9990	10000	10010	10020	10030	10040	10050	10060	10070	10080	10090
C62	10120	10130	10140	10150	10160	10170	10180	10190	10200	10210	10220	10230	10240	10250	10260
C63	10290	10300	10310	10320	10330	10340	10350	10360	10370	10380	10390	10400	10410	10420	10430
C64	10460	10470	10480	10490	10500	10510	10520	10530	10540	10550	10560	10570	10580	10590	10600
C65	10630	10640	10650	10660	10670	10680	10690	10700	10710	10720	10730	10740	10750	10760	10770
C66	10790	10800	10810	10820	10830	10840	10850	10860	10870	10880	10890	10900	10910	10920	10930
C67	10950	10960	10970	10980	10990	11000	11010	11020	11030	11040	11050	11060	11070	11080	11090
C68	11120	11130	11140	11150	11160	11170	11180	11190	11200	11210	11220	11230	11240	11250	11260
C69	11290	11300	11310	11320	11330	11340	11350	11360	11370	11380	11390	11400	11410	11420	11430
C70	11460	11470	11480	11490	11500	11510	11520	11530	11540	11550	11560	11570	11580	11590	11600
C71	11630	11640	11650	11660	11670	11680	11690	11700	11710	11720	11730	11740	11750	11760	11770
C72	11790	11800	11810	11820	11830	11840	11850	11860	11870	11880	11890	11900	11910	11920	11930
C73	11950	11960	11970	11980	11990	12000	12010	12020	12030	12040	12050	12060	12070	12080	12090
C74	12120	12130	12140	12150	12160	12170	12180	12190	12200	12210	12220	12230	12240	12250	12260
C75	12290	12300	12310	12320	12330	12340	12350	12360	12370	12380	12390	12400	12410	12420	12430
C76	12460	12470	12480	12490	12500	12510	12520	12530	12540	12550	12560	12570	12580	12590	12600
C77	12630	12640	12650	12660	12670	12680	12690	12700	12710	12720	12730	12740	12750	12760	12770
C78	12790	12800	12810	12820	12830	12840	12850	12860	12870	12880	12890	12900	12910	12920	12930
C79	12950	12960	12970	12980	12990	13000	13010	13020	13030	13040	13050	13060	13070	13080	13090
C80	13120	13130	13140	13150	13160	13170	13180	13190	13200	13210	13220	13230	13240	13250	13260
C81	13290	13300	13310	13320	13330	13340	13350	13360	13370	13380	13390	13400	13410	13420	13430
C82	13460	13470	13480	13490	13500										

Cont.

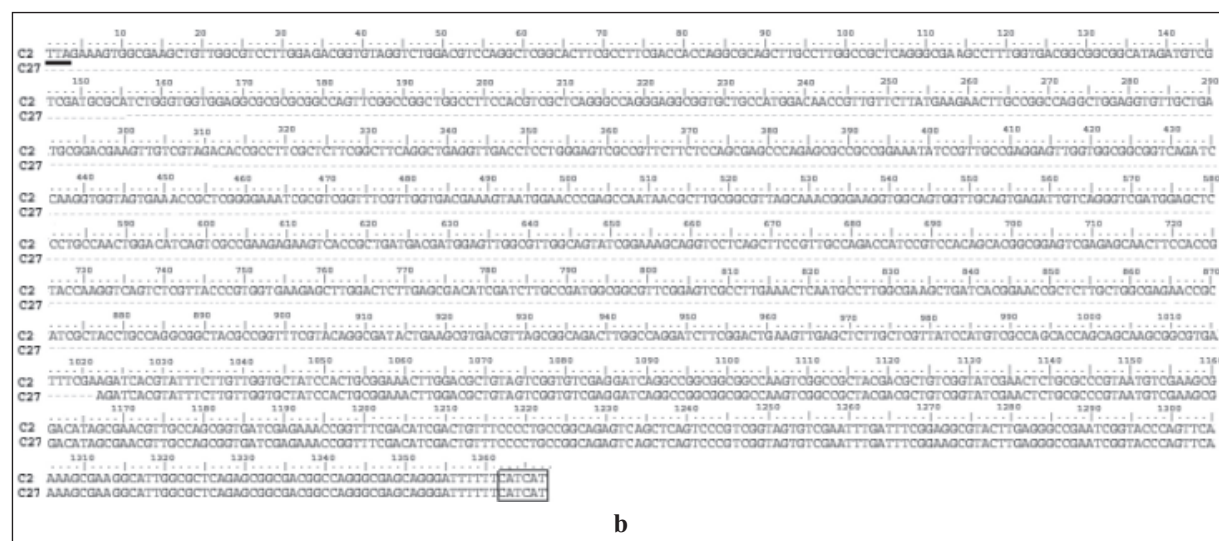


Fig. 4. Multiple alignments of *Avin_16040* gene sequences (gene direction 3' – 5') maintained in the recombinant *E. coli* DH5 α clones. Results showed truncated *Avin_16040* genes as indicated by discrepancy in the DNA insert sizes. The start and stop codons are indicated by the black striped boxes and black lines, respectively. a, clones C10 and C84 maintained 3'- and 5'-terminal sequences of *Avin_16040* gene; b, clone C27 maintained a short fragment of *Avin_16040* gene at the 5'-terminal. Complete *Avin_16040* gene consisted of 1,368 nucleotide bases.

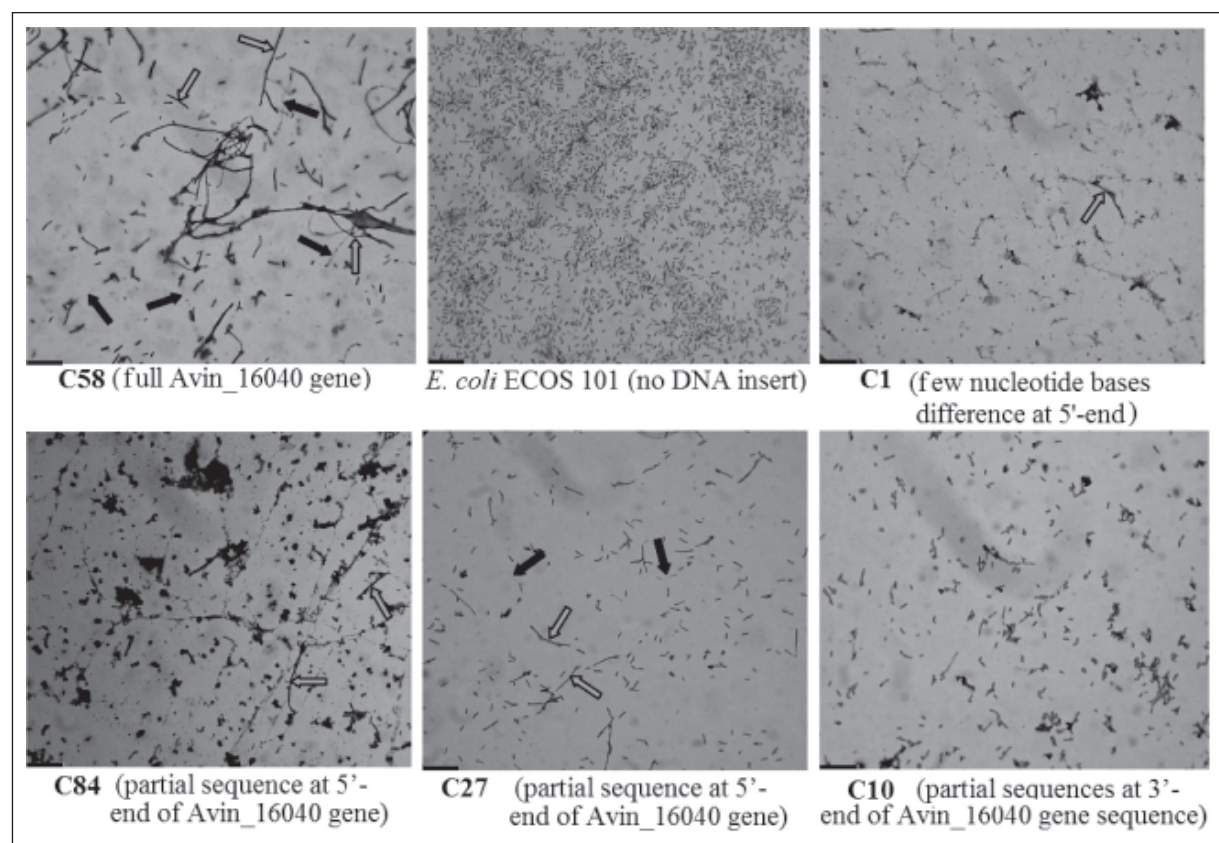


Fig. 5. Compound microscopic images of recombinant clones (Gram stained) at 1000x magnification. The block and black arrows indicate the filamentous cells and “transparent” tube-like structures resulted from the expression of *Avin_16040* gene sequences. The results suggested that the filamentous cell structure was not necessarily caused by complete gene sequence of *Avin_16040*. Instead, some recombinant *E. coli* DH5 α clones were found to maintain smaller DNA fragment of the *Avin_16040* gene but still able to acquire the filamentous cell shapes. The lost nucleotide sequences could have been caused by self-deletion or repair mechanism of the heterologous *E. coli* host. Based on the results obtained, the 5'-end of *Avin_16040* gene most possibly consisted of DNA sequence which caused the filamentous cell structures. Scale bar = 10 μ m.

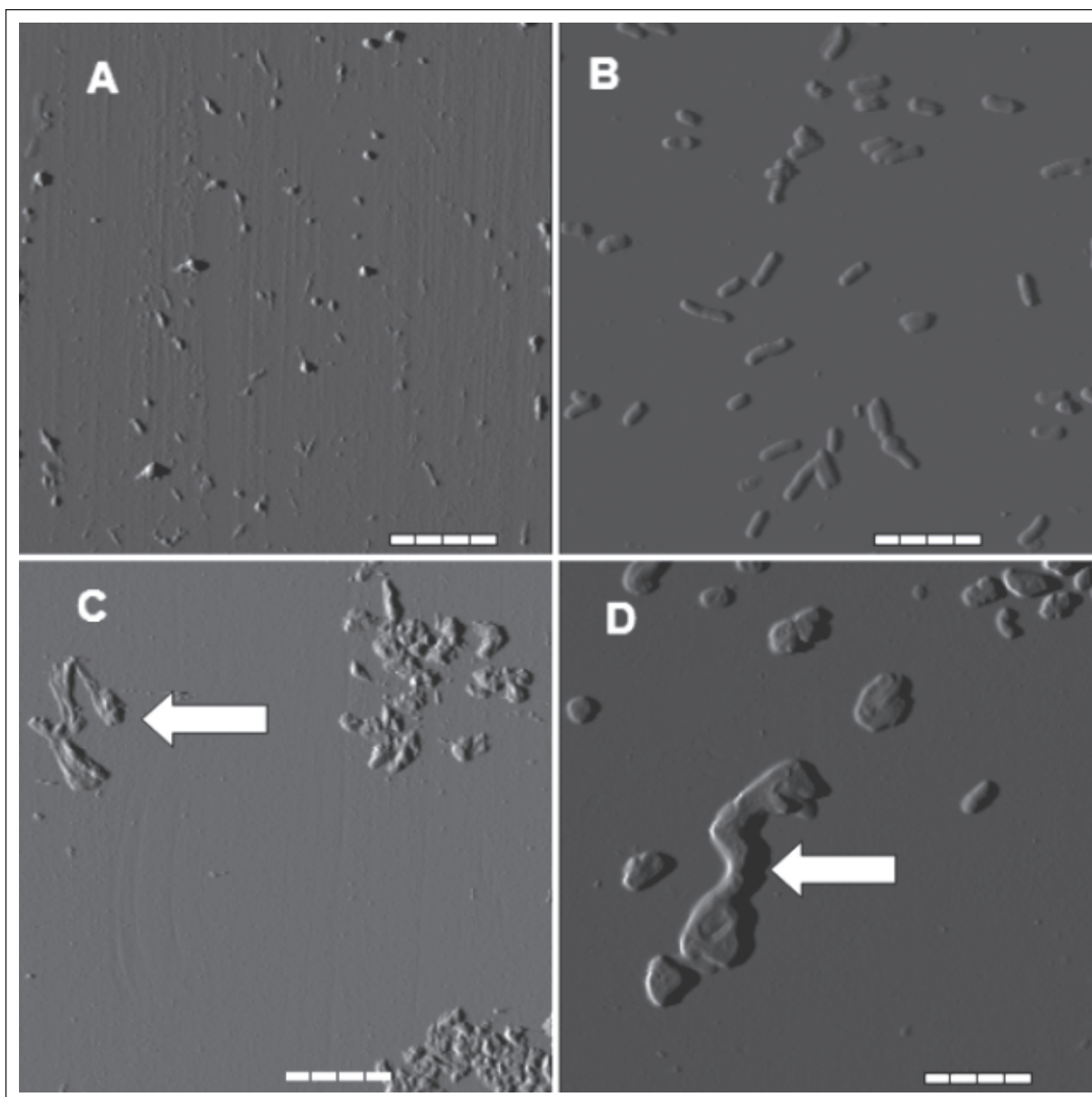


Fig. 6. Cell surface topography of *E. coli* DH5α as displayed by AFM. The images showed recombinant *E. coli* clones transformed with full gene of *Avin_16040* (C, D) have developed elongated as well as bigger cells than the untransformed wild type *E. coli* cells (A, B). During root interaction, both the non-transformed *E. coli* cells (B) and *E. coli* cells containing *Avin_16040* gene (D) displayed thicker cell wall appearances. The effect was particularly apparent for the *E. coli* clones containing *Avin_16040* gene. A, non-transformed *E. coli* cells (non-root-interaction), B, non-transformed *E. coli* cells (root-interaction), C, recombinant *E. coli* cells containing *Avin_16040* gene (non-root-interaction), D, recombinant *E. coli* cells containing *Avin_16040* gene (root-interaction). White arrows showed the elongated morphology of *E. coli* cell containing *Avin_16040* gene. Scale bar = 10 μm.

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